The Effect of the β-Human Chorionic Gonadotropin-Related Peptide (EA230) on Brain Death Induced Inflammation in Rats

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Keywords
Brain death, inflammation, liver, kidney, human chorionic gonadotropin hormone, EA230, AQGV
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Article Information
Running head: The effect of hCG-related oligopeptide EA230 on BD-induced inflammation in rats.
Word count: 7298
Word count abstract: 219
Tables: 1
Total figures: 11
Color figures: 6

Authors’ Contributions
Erp A.C. van, Rolando R.A., Leuvenink H.G., Ottens P.J. and Veldhuis S. designed and performed experiments, analyzed data and wrote the paper. Leuvenink H.G. and Ploeg R.J. edited and approved the final manuscript.

Disclosure
The authors of this manuscript have no conflicts of interest to disclose.

Funding
Funding for the project was provided by the Department of Surgery, UMCG, Groningen.

Abbreviations
BD brain death
hCG human chorionic gonadotropin
AQGV Alanine-Glutamine-Glycine-Valine
IEG Immediate Early Genes
PBS phosphate buffered saline
NA noradrenaline
(s)TNF-R (soluble) tumor necrosis factor receptor
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1 Introduction

Organ transplantation has become the treatment of choice for patients with end stage organ failure. The limiting factor in the success of organ transplantation has been the shortage of organs that meet the required criteria for transplantation [1]. At the end of 2011, more than 10,000 patients from countries in the Eurotransplant zone were on the waiting list to receive an organ graft [2]. Unfortunately, there remains a discrepancy between the number of organs available for donation and the number of organ grafts required for patients on the waiting list. In the United States alone, more than 2000 patients with end stage liver failure die each year while one the waiting list [3].

There are three main categories of organ donation: living donation, heart beating donation and non-heart beating donation. Heart beating donors are brain dead (BD) donors with an intact circulation, whereas non-heart beating donors do not have any circulation. BD-donation is the main source of organs for transplantation worldwide. However, the shortage of suitable organs combined with the increasing number of patients that require transplantation have resulted in the acceptance of extended criteria (or marginal) deceased donors and an increase in living donors [4]. In liver transplantation, recipients of marginal liver donors are at a higher risk to develop primary organ dysfunction than optimal liver donors [5]. Primary organ dysfunction leads to lower chances of graft survival and higher chances to develop more acute rejection as well as chronic transplant dysfunction [5]. In kidney transplantsations, grafts from BD donors are sub-optimal when compared to living donors. Even with a higher degree of HLA mismatching in the living unrelated transplantation, kidney grafts from living unrelated donors have higher survival rates compared to cadaver grafts [6]. But since donation after BD remains the main source of organs for transplantation, research has focussed on optimizing outcomes of transplantations with BD donors.

BD negatively effects organ quality resulting in impaired graft function and patient outcome when compared to living donation [7]. The negative effects of BD include hemodynamic instability, hormonal impairment and a cascade of inflammatory events in the donor [7,8,9,10]. Hemodynamic instability is caused by a sudden increase in intracranial pressure during BD, resulting in ischemia of the cerebrum, brain stem and spinal cord. When systemic hypertension fails to maintain cerebral perfusion, a sympathetic catecholamine storm facilitates a rise in mean arterial pressure from hypotensive to normotensive pressure (figure 5). During and following this period, donor organs can be negatively affected by ischemia and compromised organ reperfusion [1,7,18]. Ischemia of the brain results in failure of the hypothalamus and pituitary. Cessation of the hypothalamic-pituitary axis in BD thereby negatively affects the systemic hormone regulation. Levels of vasopressin, thyroid and adrenocorticotrophic hormones as well as cortisol decrease during BD, making hormonal replacement therapy a research field of great interest [9,10]. Finally, brain death is associated with systemic as well as organ-specific pro-inflammatory changes based on activation of vascular endothelium, the coagulation and complement system and the innate and adaptive immune system [7]. This pro-inflammatory environment is seen in an increased expression of cytokines such as IL-6 and TNF-α, adhesion molecules and organ-infiltrated leukocytes [7,11,12,13].

To counter hemodynamic instability such as prolonged hypotension, baseline monitoring, fluid replacement therapies and vasoactive drugs are used in brain dead patients [14]. Each individual organ requires organ-specific treatment guidelines. Research has focused on preventing, counter-acting or blocking each of these physiological processes in brain dead donors. In line with this idea, compounds able to suppress the immune system in a brain-dead setting are potentially beneficial therapeutic strategies to improve transplantation.
outcome. In mouse models of haemorrhagic shock, sepsis and diabetes, oligopeptides related to the β-subunit of human chorionic gonadotropin (hCG) were proven to exert immunosuppressive effects [15,16].

Human chorionic gonadotropin is a hormone secreted by the placenta during human pregnancy. This hormone was linked to the immune system because symptoms of Th1 mediated autoimmune diseases (cellular immune diseases), such as rheumatoid arthritis and multiple sclerosis, decline during pregnancy [17]. In a mouse model, a peptide fraction of the β-loop of hCG exhibited immunosuppressive activity by inhibiting the onset of autoimmune type I diabetes in previously non-diabetic mice [18]. In a model of septic shock, various oligopeptides of the hCG β-subunit inhibited inflammation, severity of disease and mortality in LPS-induced shock by either preventing cytokine release and transcription levels or significantly down-regulating neutrophil accumulation in the liver [18]. Of the different oligopeptides, the synthetic tetrapeptide Ala-Gln-Gly-Val (AQGV, also called EA230), showed strong immunosuppressive activity effects on early and late inflammation [19,20, figure 1]. The safety, tolerability, pharmacokinetics and pharmacodynamics of EA230 have also been successfully tested in phase I clinical trials in humans. Phase II studies are underway [21,22].

**Figure 1.** Structure of the β-loop of human chorionic gonadotropin (hCG), with the amino acid sequence of the second loop shown on the right. The arrows indicate the preferred cleavage site for the various oligopeptides [17,23].

During and following brain death, immunological factors (rejection with or without delayed-graft function) as well as non-immunologic factors are risk factors for lower graft
survival [24]. Immunogenic tissue activation occurs after brain death, indicated by an increased expression of immediate early genes (IEG), cell adhesion molecules and influx of leukocytes [25]. The influence of immunity during brain death together with the immunosuppressive qualities of oligopeptides of hCG in models of septic and haemorrhage shock, led us to combine these ideas in brain death. The purpose of this study was to investigate the effects of EA230, the oligopeptide AQGV of the β-loop of hCG, in an already established brain death model in rats. Based on the immunosuppressive qualities of oligopeptides of hCG in other models, we hypothesize that administration of EA230 results in the attenuation of inflammation as well as liver and kidney damage in brain dead donors.

2 Materials and methods

2.1 Animals

Adult, male Fisher rats (F344, 250-280 g, Harlan, United Kingdom) were used. The rats were bred under specific pathogen-free conditions and acclimated in the animal facility of the University Medical Centre of Groningen for at least one week after arrival. Rats had access to pelleted food and sterilized water ad libitum.

2.2 EA230

The selected oligopeptide Ala-Gln-Gly-Val (AQGV, EA230) was synthesized (Ansynth BV, Roosendaal, the Netherlands) using fluorencylmethoxycarbonyl (Fmoc)/tert-butyl-based methodology with a 2-chlorotritylchloride resin as the solid support. EA230 was dissolved in phosphate buffered saline (PBS) at a concentration of 1 mg/mL and stored at -20°C.

2.3 Brain-death model

The brain dead model used in this experiment was based on the gradual onset brain death model developed by Kolkert et al. [26]. Rats were anaesthetized with 5% isoflurane in a mixture with oxygen (1L/min). During brain death induction, anaesthesia was maintained with O₂/isoflurane (2%). A cannula was placed in the right femoral artery to monitor blood pressure. The right femoral vein was cannulated to provide intravenous access. Animals were intubated via a tracheostomy and continuously ventilated mechanically during the experiment. A hole was drilled frontolateral to the bregma after which a 4F Fogarty balloon catheter was inserted into the extradural space. Brain death was induced gradually in a period of 30 minutes by inflation of the balloon with 0.5 mL PBS, using a syringe pump (figure 2-5). After start of the induction, a hypotensive period and a subsequent short peak in blood pressure were followed by a rapid fall in blood pressure. Next, blood pressure slowly started to increase due to catecholamine-release. Inflation of the balloon was stopped when blood pressure rose to its basal level, while pressure in the balloon was maintained for the remainder of the experiment (figure 6). Brain death could be determined about 30 minutes after onset of
brain death and confirmed by the absence of corneal reflexes and a positive apnoea test. During the 4-hour brain death period, mean arterial pressure (MAP) was maintained above 80 mmHg (considered normotensive). If necessary, colloid infusion with polyhydroxyethyl starch (HAES, 100 g/L in saline) was started to maintain a normotensive MAP. Unresponsiveness to HAES administration indicated the start of an intravenous noradrenaline (NA) drip (1mg/mL) to maintain a normotensive MAP. The body temperature and end tidal CO$_2$ were monitored and maintained at 37 °C and between 20-22 mmHg, respectively. Esmeron (0.6 mg/kg) was administered 15 minutes before the end of brain death. The rat was then turned in supine position and the abdomen was opened. At 4 hours of brain death, 5 mL of blood and all of the urine were collected, after which all abdominal organs were flushed with 50 mL cold saline. After the flush-out, liver, kidney, jejunum, ileum, spleen, heart and lung were harvested and tissue samples were snap frozen in liquid nitrogen and stored at -80 °C or fixated in 4% paraformaldehyde. Centrifuged blood samples and urine from the bladder were also snap frozen.

Figure 2-3. Insertion of a 4F Fogarty balloon catheter was inserted into the extradural space frontolateral to the bregma.

Figure 4-5. Brain death induction by inflation of a Fogarty balloon catheter in a period of 30 minutes with 0.5 mL PBS, using a syringe pump.
Figure 6. Mean arterial pressure (MAP) during brain death induction. Following balloon insertion, the MAP shortly rises after insertion of the balloon, followed by quick drop in MAP and a subsequent hypotensive period. After about 30 minutes, blood pressure becomes normotensive again.

Rats were randomly divided, each group consisting of 8 animals (n=8). Sham-operated rats served as controls accounting for the surgical procedure. These sham-animals were treated according to the brain death model, up until the insertion of the Fogarty catheter balloon. After a hole was drilled, animals were ventilated for half an hour under anaesthesia before they were sacrificed. Rats in the brain death group were treated according to the brain death model described above. After cannulation of the femoral artery and vein, rats were injected with PBS, EA230 (10 mg/kg) or EA230 (30 mg/kg) intravenously, 30 minutes before the start of the brain death induction.

The animals were randomly assigned to one of six experimental groups:

1. Sham + PBS
2. Sham + EA230 10 mg/kg
3. Sham + EA230 30 mg/kg
4. BD + PBS
5. BD + EA230 10 mg/kg
6. BD + EA230 30 mg/kg
2.4 Plasma determinations

Plasma levels of creatinine, lactate dehydrogenase (LDH), alanine transaminase (ALAT), aspartate transaminase (ASAT) and bilirubin were determined at the clinical chemistry lab of University Medical Centre Groningen according to standard procedures.

Plasma levels of IL-6 were determined by IL-6 enzyme linked immunosorbent assay (ELISA) (R&D Systems Europe Ltd. Abingdon, Oxon OX143NB, UK) according to the manufacturer’s instructions. All samples were analysed in duplicate and read at 450nm.

2.5 RNA isolation and cDNA synthesis

Total RNA was isolated from whole kidneys and livers using TRIzol (Life Technologies, Gaithersburg, MD). Absence of DNA contamination in the RNA samples was verified by means of a RT-PCR reaction without adding reverse transcriptase, using GAPDH primers. For cDNA synthesis, 1 μl T11VN Oligo-dT (0,5 μg/μl) and 1 μg mRNA were incubated for 10 min at 70 °C and cooled directly afterwards. cDNA was synthesized by adding a mixture containing 0,5 μl RNaseOUT® Ribonuclease inhibitor (Invitrogen, Carlsbad, USA), 0,5 μl RNase water (Promega), 4 μl 5 x first strand buffer (Invitrogen), 2 μl DTT (Invitrogen), 1 μl dNTP’s and 1 μl M-MLV reverse transcriptase (Invitrogen, 200U). The mixture was kept at 37 °C for 50 min. Reverse transcriptase was inactivated by incubating the mixture at 70 °C for 15 min. Samples were stored at -20 °C.

2.6 Real-Time PCR

Fragments of several genes were amplified with primer sets outlined in Table 1. Pooled cDNA obtained from brain-dead rats was used as an internal reference. Gene expression levels were quantified by normalization against the mRNA levels of β-actin. Real-time PCR was carried out in reaction volumes of 15 μl containing 10 μl of SYBR Green mastermix (Applied biosystems, Foster City, USA), 0.4 μl of each primer (50 μM), 4.2 μl of nuclease free water and 10 ng of cDNA. All samples were analysed in triplicate.

Table 1. Primer sequences used for Real-Time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers</th>
<th>Amplication size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6</td>
<td>5'-CCAACTTCCAATGCTCTCTCTATG-3'</td>
<td>89</td>
</tr>
<tr>
<td>MCP-1</td>
<td>5'-TTCAAGTGCTTTCAAGAGTTGGAT-3'</td>
<td>78</td>
</tr>
<tr>
<td>TNF-α</td>
<td>5'-CTTTGAATGTGAACCTTGACCCATAA-3'</td>
<td>79</td>
</tr>
<tr>
<td></td>
<td>5'-ACAGAAAGTGGCTTTGAGTGTTGT-3'</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5'-GGCTGCTTTGGTTCAGATGT-3'</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5'-CAGGTTGGAGCAAACCTACAGTT-3'</td>
<td></td>
</tr>
</tbody>
</table>
Thermal cycling was performed on the Taqman Applied Biosystems 7900HT Real Time PCR System with a hot start at 50 °C for 2 min, followed by 10 min at 95 °C. The second stage was started at 95 °C for 15 s (denaturation step) and followed by 60 s at 60°C (annealing step and DNA synthesis). The latter stage was repeated 40 times. The third stage was included to detect formation of primer dimers (melting curve) and began with 15 s at 95 °C. Primers were designed with Primer Express software (Applied Biosystems) and primer efficiencies were tested by a standard curve for the primer pair by means of the amplification of serially diluted cDNA samples (10 ng, 5 ng, 2.5 ng, 1.25 ng and 0.625 ng) obtained from brain-dead rats. PCR efficiency was found to be $1.8 < \varepsilon < 2.0$. Real time PCR products were checked for product specificity on a 1.5% agarose gel. Results were expressed as $2^{\Delta\Delta CT}$ (CT: Threshold Cycle).

2.7 Immunohistochemistry

To detect polymorphonuclear cells (PMNs) in kidney and liver, immunohistochemistry was performed on 5-μm tissue cryosections. Sections were fixated for 10 min using acetone and then stained with HIS-48 mAb (supernatant, two times diluted), using an indirect immunoperoxidase technique. Endogenous peroxidase was blocked using H$_2$O$_2$ 0.01% in phosphate-buffered saline for 30 min. After thorough washing, sections were incubated with horseradish peroxidase-conjugated rabbit anti-mouse IgG as a secondary antibody for 30 min, followed by incubation with a tertiary goat anti-rabbit IgG antibody for 30 min (both from Dako, Glostrup, Denmark). The reaction was visualized using 9-amino-ethylcarbazole as chromogen and H$_2$O$_2$ as substrate. Sections were counter stained using Mayer hematoxylin solution (Merck, Darmstadt, Germany). Negative antibody controls were performed. Localization of immunohistochemical staining was assessed by means of light microscopy. For each tissue section, positive cells per renal glomerulus and renal interstitium in the cortex and liver were counted in 10 microscopic fields at 20x magnification.

2.8 In vitro stimulation of rat leukocytes with lipopolysaccharide

Heparinised blood (3-4 ml) was drawn from the inferior vena cava of adult, male Fisher rats (F344, 250-280 g, Harlan, United Kingdom) and mixed with 45 mL of ammonium chloride solution. After storage at 4 °C for 15 minutes, the solution was centrifuged at 1000G for 5 minutes. The leukocytes in the pellet were separated from the supernatant with the lysed red blood cells and then mixed with RPMI-1640 +10% Fetal Bovine Serum (FBS) and counted with a cell-counter. Lipopolysaccharides from *Escherichia coli* 0111:B4 (L3491, Sigma-Aldrich) was diluted in phosphate buffered saline (PBS) at a concentration of 1 mg/mL. EA230 was diluted in phosphate buffered saline at a concentration of 10 mg/mL. The leukocytes were added at 1 x 10$^6$/mL/well or 2 x 10$^6$/mL/well in 48 well plates with RPMI-1640 +10% FBS medium. Then, 100 ng/mL, 500 ng/mL or 1000 ng/mL LPS was added. EA230 was added before, during or after LPS stimulation in concentrations of 20 μg/mL or 50 μg/mL. 5, 12, 18 or 24 hours after LPS administration, supernatant was collected from each well and stored at -20°C until cytokine measurement. (figure 7). Leukocytes with medium alone and leukocytes with medium and LPS or EA230, were used as controls and were collected at the same time point as the other samples.
Plasma levels of IL-6 were determined by IL-6 enzyme linked immunosorbent assay (ELISA) (R&D Systems Europe Ltd. Abingdon, Oxon OX143NB, UK) according to the manufacturer’s instructions. All samples were analysed in duplicate and read at 450nm.

![Diagram](image)

**Figure 11.** General overview of *in vitro* experiment setup

### 2.9 Statistical analysis

Due to the two-factorial design of the experiment, the two-way ANOVA test was done to analyse the results (SPSS Statistics 20). After data transformations, data were normally distributed and variances of the dependent variable equal across groups according to Levene’s test of equality of error variances (indicated by P>0.05). This allowed us to use the two-way ANOVA test despite the small group sizes.

Plasma bilirubin levels (both total and direct bilirubin) did not have homogeneous variances. For these parameters, the Kruskal-Wallis test was performed to analyse between the six experimental groups, followed by the Mann-Whitney test to compare between two groups individually (Prism 5.0 GraphPad).

All statistical tests were 2-tailed and *p* < 0.05 was regarded as significant. Results are presented as mean ± SEM (standard error of the mean).

### 3 Results

#### 3.1 Brain death induction

The mean arterial blood pressure in the rat slightly increased immediately after the start of brain death induction, followed by a drop and subsequent increase. This pattern of the MAP during the BD induction was seen uniformly and consistently among all BD groups, with a mean of 31.9 minutes for the total induction (figure 8). After completing the induction, all 32 animals (n=8 per group) were kept at a MAP of at least 80 mmHg during the experiment. In saline treated brain-dead rats, infusion of 0.81 ± 0.59 ml 10% HAES was required to maintain stable blood pressure. In EA230 (30 mg/kg) treated BD rats, infusion of 1.13 ± 0.35 ml 10% HAES was required to maintain stable blood pressure (*p* = 0.315 vs. saline treatment). Saline-treated BD rats required 0.83 ± 0.95 ml NA and EA230-treated BD rats required 0.69 ± 0.83 ml NA (*p* = 0.874 vs. saline treatment).
3.2 Organ function

3.2.1 Kidney

Renal function was assessed measuring plasma creatinine levels. Plasma creatinine levels significantly increased in brain dead animals compared to sham-operated animals ($p < 0.01$, figure 8). Amongst the six groups, no significant differences were found between saline and EA230 treatment groups (figure 8).

3.2.2 Liver

Cellular liver damage and liver function were determined measuring plasma aspartate (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH) and direct and total bilirubin levels. Plasma AST, ALT and LDH levels significantly increased in brain dead animals compared to sham-operated animals ($p < 0.05$, figure 8).

Plasma direct and total bilirubin levels in EA230-treated BD animals showed no differences compared to saline-treated BD animals. Plasma total bilirubin levels in saline-treated BD animals significantly increased compared to saline-treated sham animals ($p = 0.005$, figure 8). Plasma direct bilirubin levels in saline-treated BD and sham animals were not significantly different. Amongst the six groups, no significant differences in AST, ALT and LDH were found between saline and EA230 treatment groups (figure 8).

3.3 Inflammatory status

3.3.1 Plasma levels of Interleukin-6

The systemic inflammatory status of the rats was assessed looking at plasma levels of pro-inflammatory cytokine interleukin-6 (IL-6). Plasma IL-6 levels significantly increased in brain dead animals compared to sham-operated animals ($p < 0.05$, figure 2). Amongst the six groups, no significant differences were found between saline and EA230 treatment groups (figure 9).

3.3.2 Renal gene expression levels

The renal inflammatory status was assessed looking at gene expression of pro-inflammatory cytokines and acute phase reactants IL-6, tumour necrosis factor-6 (TNF-α) and monocyte chemotactic protein-1 (MCP-1). Plasma IL-6 and MCP-1 and TNF-α levels significantly increased in brain dead animals compared to sham-operated animals ($p < 0.01$, figure 9). Amongst the six groups, no significant differences in IL-6, MCP-1 and TNF-α levels were found between saline and EA230 treatment groups (figure 9).
3.3.3 Hepatic gene expression levels

The hepatic inflammatory status was assessed looking at gene expression of pro-inflammatory cytokines and acute phase reactants IL-6, TNF-α and MCP-1. Plasma IL-6 and MCP-1 significantly increased in brain dead animals compared to sham-operated animals ($p < 0.01$, figure 9). Amongst the six groups, no significant differences in IL-6 and MCP-1 levels were found between saline and EA230 treatment groups (figure 9).

Plasma TNF-α significantly decreased in brain dead animals compared to sham-operated animals ($p < 0.01$, figure 9). Amongst the six groups, no significant differences in TNF-α levels were found between saline and EA230 treatment groups (figure 9).

3.4 Polymorphonuclear influx

The influx of polymorphonuclear (PMN) cells in the kidney and liver was determined using tissue cryosections stained with HIS-48 mAb. The number of positive cells in de glomeruli of the kidney per microscopic field was $1.14 \pm 0.28 \text{ mm}^2$ in sham-operated controls and did not change significantly in BD groups.

The number of positive cells per microscopic field in de glomeruli and interstitium of the kidney and the liver significantly increased in brain dead animals compared to sham-operated animals ($p < 0.01$ for both groups, figure 10). Amongst the six groups, no significant differences in number of positive cells in the glomeruli and interstitium of the kidney were found between saline and EA230 treatment groups (figure 10).

In the glomerulus of the kidney, the number of positive cells per microscopic field were not significantly different in BD animals compared to sham-operated animals ($p = 0.595$, figure 10). Amongst the six groups, no significant differences in number of positive cells in the interstitium of the liver were found between saline and EA230 treatment groups (figure 10).
Figure 8. Plasma levels of interleukin-6 (IL-6) to assess the systemic inflammatory status and lactate dehydrogenase (LDH), aspartate aminotransferase (AST), alanine aminotransferase (ALT), total and direct bilirubin to assess renal and hepatic function and damage, following 4 hours of brain death (BD) in rats pre-treated with EA230 or phosphate buffered saline (PBS) solution. A) IL-6 plasma levels significantly increased in BD animals compared to sham animals. B) Creatinine plasma levels significantly increased in BD animals compared to sham animals. C) and D) ASAT and total bilirubin levels in saline-treated BD animals significantly increased compared to saline-treated sham animals (p=0.027 for ASAT and p=0.005 for total bilirubin)
Figure 9. Renal and hepatic interleukin-6 (IL-6), monocyte chemotactic protein-1 (MCP-1) and tumour necrosis factor-α (TNF-α) gene expression following 4 hours of brain death (BD) in rats pre-treated with EA230 or phosphate buffered saline (PBS). A) and B) No differences in gene expression amongst BD groups. C) TNF-α expression in saline-treated BD animals not significantly different than sham-animals, EA230 (30 mg/kg)-treated BD animals with significant higher levels of TNF-α compared to sham animals. D) and E) No significant differences in gene expression amongst BD groups. F) TNF-α expression in saline and EA230 (10mg/kg)-treated BD animals significantly lower than sham-operated animals. TNF-α expression in EA230 (30mg/kg)-treated BD animals significantly higher than saline-treated BD animals (p = 0.031).

* p < 0.01
Figure 10. Renal (glomeruli and interstitium) and hepatic leukocyte infiltration following 4 hours of brain death (BD) in rats pre-treated with EA230 or phosphate buffered saline (PBS). A) No significant differences in leukocyte infiltration in the kidney glomeruli were found BD animals compared to sham animals. Also no significant differences amongst EA230 treatment groups. B) and C) Leukocyte infiltration in kidney interstitium and liver interstitium increased in BD animals compared to sham animals (*p < 0.01).

3.5 In vitro, LPS-stimulated rat leukocytes and EA230 treatment

LPS dosage and timing of LPS stimulation was determined to be 500 ng/mL and 12 hours with an average IL-6 concentration of 1538.7 ± 85.7 pg/mL (standard deviation). Lowest IL-6 levels were found when EA230 was added 30 minutes after LPS addition. Optimal EA230 concentration was 20 μg/mL with an IL-6 level of 270.3 ± 31.0 pg/mL (figure 11). Treatment with EA230 before LPS addition (pre-treatment) and treatment with EA230 after LPS addition (post-treatment) was not significantly different (p = 0.180). Finally, no significant different were found when comparing pre-treatment with EA230, or post-treatment after 30 minutes, 30 minutes and 4 hours, 4 hours or with a double dose of EA230 after 4 hours (p = 0.406). Control LPS concentration was significantly higher than EA230 without LPS, or no LPS or EA230 at all (p = 0.0498, figure 11).
Figure 11. Interleukin-6 (IL-6) levels in rat leukocytes treated in vitro with lipopolysaccharide (LPS) and/or EA230. A) Optimal dosage and timing of LPS stimulation were 500 ng/mL and 12 hours with an average of IL-6 of 1538.7 ± 85.7 pg/mL. B) EA230 (20 μg/mL) added 30 minutes after LPS addition was optimal with an IL-6 level of 270.3 ± 31.0 pg/mL. C) No significant differences in IL-6 levels with pre- or post-treatment with EA230 (p = 0.180) were found. D) Control groups with LPS were significantly higher than groups with EA230 alone or just cells (p = 0.0498). E) No significant different between pre-treatment with EA230, or post-treatment after 30 minutes, 30 minutes and 4 hours, 4 hours or with a double dose of EA230 after 4 hours (p = 0.406).
4 Discussion

In this study, we used a brain death animal model to determine the effects of AQGV (10mg/kg or 30mg/kg, administered intravenously 30 minutes prior to brain death induction) on hemodynamic and inflammatory status, organ function and leukocyte infiltration in brain dead rats. AQGV has previously been shown to have anti-inflammatory effects in models of septic and hemorrhage shock [18,20].

When looking at the hemodynamic profile, all rats showed a short rise, followed by the characteristic drop in blood pressure following brain death induction. The quick rise in blood pressure reflects the body’s attempt to maintain cerebral perfusion, followed by the period of hypotension when mean arterial pressure does not suffice to maintain cerebral perfusion. The rise back to normotensive blood pressure is facilitated by a sympathetic catecholamine storm [1]. No differences in hemodynamic profiles were seen between the saline-treated and EA230-treated animals.

To determine the effect of brain death on organ function, function and damage parameters of the kidney and liver were assessed. Plasma levels of creatinine, aspartate aminotransferase (AST), alanine aminotransferase (ALT) and lactate dehydrogenase (LDH) have been shown to increase during brain death in various rat models [27,28,29]. Plasma creatinine levels were significantly increased in brain dead animals compared to sham-operated animals, indicating reduced kidney function in brain dead animals. Plasma aspartate (AST) and total bilirubin levels significantly increased in brain dead animals compared to sham animals (p = 0.027 and p = 0.005, respectively). Increased AST levels can indicate acute liver damage, though it is not liver specific. The results regarding the bilirubin levels conclude a rise in indirect, or unconjugated, bilirubin. Brain death related events such as hypoxia, congestive heart failure or haemolysis might cause hyperbilirubinemia. However, administration of EA230 did not improve renal or hepatic function and injury nor damage parameters.

Interleukin-6 is an important inflammatory marker because its induction leads to an increase in the expression of acute phase genes. The acute phase response of an organism is intended to restore disturbances in the physiological homeostasis, resulting in both local and systemic reactions. Accumulation and activation of PMNs and mononuclear cells at the site of injury are examples of local reactions of the innate immune response [30]. Plasma IL-6 levels were significantly higher in BD animals compared to sham-operated animals, confirming the link between brain death and inflammation. Research performed in both human and animal settings shows an increase in inflammatory cytokines during brain death [11,12,13,31]. In transplantation of organ grafts from brain-dead donors, an increased level of IL-6 before organ retrieval even negatively affects the long-term outcome for the recipient following transplantation [32].

The gene expression of IL-6, monocyte chemotactic protein-1 (MCP-1) and tumour necrosis factor-α (TNF-α) was determined to assess organ-specific inflammation. MCP-1 is a cytokine that is involved in the recruitment of monocytes and T cells. TNF-α plays an important role in the pathogenesis of inflammation, septic shock, tissue injury and cachexia [33]. Gene expression of IL-6 and MCP-1 in the liver and kidney were significantly upregulated in the brain dead setting. Interestingly, TNF-α gene expression was not significantly different in the kidneys and even decreased in the livers of brain dead rats when compared to sham animals. A decrease of TNF-α gene expression in brain dead animals is in contrast with findings of other research [34,35]. The function of TNF and the TNF-receptors (TNF-Rs) in the pathogenesis of disease remain unclear because it has been shown both
protective and pathogenic [36,37]. The expression of TNF-Rs is modulated by many agents, including cytokines like IL-6, hormones like thyroid stimulating hormone (TSH) and TNF itself. The complex physiological state that is BD might influence the expression of TNF-Rs. Shedding of TNF-R receptors (sTNF-Rs), induced by for example TNF or other cytokines like IL-6, are also able to compete with TNF for binding with TNF-Rs. Therefore, differences in sTNF-Rs levels might influence the effect of TNF-α during BD [36].

Leukocyte infiltration in the organ reflects a local inflammatory response. During brain death, we found an increase in leukocyte invasion in the renal interstitium, but not in the glomeruli. These data correspond to the findings of Nijboer et al. [36,37]. Leukocyte infiltration in the liver significantly increased during brain death when compared to controls. However, treatment with EA230 did not affect leukocyte infiltration.

Combining all these results, pre-treatment of BD rats with EA230 did not alter plasma cytokines nor kidney and liver damage markers and inflammatory cytokines. Therefore, we determined in vitro whether timing of EA230 administration in relation to activation of immune cells such as leukocytes might explain the lack of immunosuppressive qualities of EA230 in the BD setting. We hypothesized that EA230 might have a short incubation time, or might only work on cells that are activated by a certain stimulus, like LPS. When performing the in vitro experiment, no significant differences were found between the group pre-treated with EA230 before and the group treated with EA230 after the LPS stimulus. Also, no significant differences were found when leukocytes were treated at multiple time points after LPS addition, at a later time point and at a later time point with a double dose of EA230. Therefore, we conclude that in LPS-activated rat leukocytes, EA230 treatment was unable to reduce IL-6 levels. In other animal models that use EA230, both pre- and post-treatment with EA230 showed significantly lower cytokine levels or improved survival rates. In a rat model of haemorrhagic shock and resuscitation, rats treated with EA230 after 60 minutes of haemorrhage shock showed decreased levels of inflammatory cytokines [18]. In a mouse model of ischemia-reperfusion, administration of AQGV prior to and at the end of the ischemic period, showed significantly improved survival rates [19].

This suggests that the ineffectiveness of EA230 might be related to the hemodynamic, hormonal or inflammatory changes of BD. Marten van der Zee et al suggest that LQGV, a peptide closely related to AQGV (EA230), exerts its anti-inflammatory effects through corticosterone production by activation of the adrenal gland in mice [14]. Armelle Nicolas-Robin et al. show that 87% of BD patients suffered from adrenal insufficiency. In all patients, ACTH concentration was lower than the normal clinical range [38,39]. Juan Sebastian Danobeitia et al. show that cortisol levels in brain death non-human primates increase 30 minutes after brain death induction and subsequently decrease below baseline levels after 6 hours [12]. Lack of hypophyseal stimulation of the adrenal gland as a result of BD, or the incidence of adrenal insufficiency in BD patients, might explain why EA230 did not attenuate inflammation in our BD model. More research into the working mechanism of EA230 will need to be conducted in order to answer the question why EA230 is not effective in the BD setting.
5 Conclusion

EA230 shows strong immunosuppressive effects in animal models of septic and haemorrhage shock. In our model, EA230 did not display these immunosuppressive effects, as it was unable to attenuate inflammation and liver- and kidney damage in BD rats. We suggest that the lack of immunosuppressive qualities of EA230 in our BD model is related to the hemodynamic, hormonal or inflammatory changes of BD. A better understanding of the working mechanism of EA230 in a BD setting might explain the inefficacy of EA230 in our model.
6 Abstract (English)

Organ transplantation has become the treatment of choice for patients with end stage organ failure. However, the shortage of donor organs has limited the success of transplantation. Donation after brain death (DBD) is the main source of organs for transplantation. Brain death (BD), however, negatively affects organ quality and function due to hemodynamic, hormonal and inflammatory changes. We hypothesized that EA230, an oligopeptide of the β-loop of human chorionic gonadotropin (hCG), would attenuate BD-induced inflammation and improve kidney- and liver damage in an already established BD-model in rats. Plasma creatinine, ASAT, total bilirubin and IL-6, as well as renal and hepatic gene expression levels of IL-6 and MCP-1, were significantly higher in BD rats compared to sham-operated rats. Polymorphonuclear (PMN) influx in kidney and liver interstitium was also significantly higher in BD rats than sham animals. No significant differences were found between EA230- and PBS-treated animals in plasma levels of creatinine, AST, ALT, LDH, direct and total bilirubin and IL-6, in gene expression levels of IL-6, TNF-α and MCP-1, or PMN influx in the kidney or liver. In vitro results show that pre- or post-treatment of LPS-activated rat leukocytes do not affect IL-6 levels. Based on these results, we suggest that the ineffectiveness of EA230 might be related to the systemic changes related to BD in our rat model.

7 Abstract (Dutch)

Orgaan transplantatie is de eerste keus bij patiënten die lijden aan ernstig organ falen. Echter, het tekort aan donoren heeft het succes van transplantatie aan banden gelegd. Donatie na hersendood is de grootste bron van organen voor transplantatie. Hersendood zorgt er echter voor dat kwaliteit en functie van de organen achteruit gaan door hemodynamische, hormonale en inflammatoire veranderingen tijdens hersendood. Wij stelden dat EA230, een oligopeptide van de β-ring van humaan choriongonadotrofine (hCG), de door hersendood-geïnduceerde inflammatie en nier- en leverschade zou tegengaan. Plasma niveaus van creatinine, ASAT, totaal bilirubine en IL-6, en renale en hepatische gen expressie van IL-6 en MCP-1, waren significant verhoogd in hersendode ratten in vergelijking met controles. Er zijn geen significante verschillen gevonden tussen EA230- en PBS-behandelde groepen in plasma niveaus van creatinine, AST, ALT, LDH, direct en totaal bilirubine en IL-6, in gen expressie van IL-6, TNF-α en MCP-1, of bij de PMN influx in de nier of de lever. In vitro resultaten laten zien dat voor- of nabehandeling van LPS-geactiveerde rat leukocyten IL-6 niveaus niet significant veranderd. Op basis van deze resultaten verwachten wij dat de ineffectiviteit van EA230 mogelijk verklard kunnen worden door de systemische veranderingen die hersendood te weeg brengt in de ratten in ons model.
8 References